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The cytolytic enzyme produced by *Bacillus carotovorus* and certain other soft rot bacteria.

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An account of our studies upon the soft rot of the carrot caused by *Bacillus carotovorus* was published in this journal three years ago¹⁾. The following statements occur therein.

„Wahrscheinlich werden weitere Studien über die bakterielle Weichfäule von Vegetabilien zu der Entdeckung vieler ähnlicher Organismen führen, von denen jeder fähig ist, eine größere Reihe von Wirtspflanzen zu befallen. Es ist sicherlich von Wichtigkeit, sowohl vom Gesichtspunkt der abstrakten Bakteriologie, als von der Pflanzenpathologie aus, daß sie vollständig bekannt werden. Die mikroskopische Untersuchung des faulenden Möhrengewebes hat gezeigt, daß der Organismus in die Interzellularräume eindringt und sich darin mit ungeheurer Schnelligkeit vermehrt. Die mittleren Lamellen der ausliegenden Zellen erscheinen erweicht oder zerstört durch die Ausscheidung von Extrakten der Bakterien, denn in ergriffenen Geweben findet Isolierung der Zellen statt. Das Protoplasma in solchen isolierten Zellen ist zusammengefallen, aber man hat keine Bakterien in den Zellen von frisch desorganisiertem Gewebe bemerkt. Diese Zerstörung der Interzellularsubstanz rührt wahrscheinlich von einem Enzym von der Natur der Cytase her“

The correctness of the first suggestion has already received ample proof both from further unpublished studies on soft rot organisms in our own laboratory and from several publications dealing with these,

1) Centralbl. f. Bakt. Abt. II. Bd. VII. 1901. p. 12 and 61. For a more detailed account of the same see Report Vermont Experiment Station. XIII. 1900. p. 299.

The present publication deals with a direct continuation of the earlier studies and the reader is referred to the former publication for details relative to the source and character of the organism, culture methods, etc.

Most of the results here given were embodied in a paper read Dec. 1902 before the Society for Plant Physiology and Morphology, Washington, D. C. Since the work was undertaken papers have been published by Spieckermann, Van Hall and Potter dealing with some of the same questions. Reference to these is made later in our text. Since our own studies were largely completed before these came to our hands it follows that the conclusions where they are identical are the more firmly established. Where our results differ from any of theirs we have carefully repeated the work and have satisfied ourselves of the correctness of our position.

This work forms one part of a comparative study of various soft-rot bacteria which has been conducted in cooperation with Messrs. H. A. Harding and F. C. Stewart of the Geneva, N. Y. Experiment Station and W. J. Morse of our institution. The full details are reserved for a joint publication from these two stations to be made soon.

Much of the study was carried on by the author while in residence at the University of Michigan and he gratefully acknowledges advice from Prof. F. C. Newcombe and also excellent help in many of the details from two student assistants, Messrs. H. D. Bone and L. P. Sprague in the work done in his own laboratory.

including those of Townsend¹⁾, Harding and Stewart²⁾ and Harrison³⁾ in America and Spieckermann⁴⁾, Van Hall⁵⁾ and Appel⁶⁾ in Europe.

A fuller understanding of the wall-dissolving or other enzymes produced by these bacteria is desirable for two reasons. First, because the parasitism in each case seems dependent upon enzyme production; second, because these organism are so similar as to make the question of their specific relationship an extremely complex one which must be settled largely by appeal to physiological characters and it would seem that enzyme production might prove of value for such differential purposes.

It is also of general interest to learn whether or not these bacterial cytolytists are identical with the cytolytic enzymes known to be developed by certain fungi, germinating seeds and pollen tubes. This latter question has been clearly raised by Green⁷⁾, who entertains the belief that these so-called "cytases" include two distinct kinds of enzyme.

The action of the organism upon the tissues of the host plant.

This was discussed in our earlier publications. Our later studies have confirmed the earlier conclusions that the action leads to the death of the cells and to the solution of the middle lamellae of the parenchymatous tissues and leaves an undissolved residual wall through which the organisms do not pass. This strictly intercellular invasion is in accord with observations made by Spieckermann and Van Hall upon their similar organism and also by Appel but is opposed to that of Potter⁸⁾ who reports penetration of the cell cavities.

As seen by the naked eye the invasion is marked by a rapid soft rot of the parenchymatous tissues, accompanied by a water-logged appearance and often with discoloration. The boundary between invaded and sound tissues is always clearly defined. Cuticularized and lignified tissues are unaffected.

Under the microscope the collapse and evident death of the protoplasm is seen to occur promptly, and co-incidentally with the following changes in the cell-membrane. There is a loss of refractiveness in the

1) Townsend, C. O., An unpublished paper on a bacterial soft rot of the Calla. Read before the Society for Plant Physiology and Morphology, New York. Dec. 1901); A soft rot of Calla Lily. (U. S. Dept. Agric. Bureau Plant Industry. Bulletin 60. June 30, 1904.)

2) Harding, H. A. and Stewart, F. C., A bacterial soft-rot of certain cruciferous plants and *Amorphophallus simlense*. (Science. N. S. Vol. XVI. 1902. p. 314.)

3) Harrison, F. C., Preliminary report on a new organism producing rot in Cauliflower and allied plants. (Science. N. S. Vol. XVI. 1902. p. 152.)

4) Spieckermann, A., Beitrag zur Kenntnis der bakteriellen Wundfäulnis der Kulturpflanzen. (Landw. Jahrb. von Thiel. 1902. p. 155.)

5) Van Hall, C. J. J., Das Faulen der jungen Schöcklinge und Rhizome von *Iris florentina* und *Iris germanica*. (Zeitschr. f. Pflanzenkr. Bd. XIII. 1903. p. 129.)

6) Appel, Otto, Untersuchungen über die Schwarzbeinigkeit und die durch Bakterien hervorgerufene Knollenfäule der Kartoffel. (Arb. a. d. biol. Abt. f. Landw. u. Forstw. am Kais. Gesundheitsamt. Bd. III. 1903. p. 364.)

7) Green, J. Reynolds, The soluble ferments and fermentation. 2. edition. 1901. p. 105.

8) Potter, M. C., On the parasitism of *Pseudomonas destructans*. (Proc. Roy. Soc. London. Vol. LXX. 1902. p. 393.)

inner lamellae of the membrane (i. e. the portion lying between middle lamella and cell cavity) which is accompanied by its strong swelling, often to twice the original thickness, and the appearance of delicate lamination.

The middle lamella scarcely changes its refractive character and is therefore brought out in sharp contrast with the inner lamellae. Neither does it swell, but instead it soon melts away, disappearing first in its thinner parts while the thickened masses at the angles of the cells persist longest but ultimately dissolve also.

Before the latter are all dissolved the cells lose their cohesion, i. e. the tissues are fully rotten. Thin sections of carrot or turnip tissue immersed in culture broths or the enzyme solutions (to be described later) generally pass through the above changes in from ten minutes to one hour's time. The disappearance of the middle lamella marks the completion of all visible action. The swollen remains of the inner lamellae are not further acted upon even after many days and give the characteristic cellulose reaction with chlor-zinc-iodide.

In order to follow some of these details more carefully, blocks of carrot and of turnip tissue in process of invasion by the organism were fixed in absolute alcohol, imbedded, sectioned and stained in various ways. These have shown that the swelling of the walls and the partial solution of the middle lamella occurs some distance, often ten cells, in advance of the actual invasion by the organism. Such sections have also shown that the middle lamella like the inner lamella shows a laminated structure when partially dissolved.

Briefly, then, the action consists primarily in the rapid and complete solution of the middle lamella, or pectic layer, of the wall. This is accompanied by the swelling of the inner lamellae, or hemicellulose layers and the revelation of a laminated structure in them which is evidently due to the occurrence of soluble pectic layers alternating with the insoluble hemicellulose layers. The hemicellulose residuum although evidently softened still serves to debar the organisms from entrance into the cell cavities, no organisms having been observed in the interior of either the cells or the vessels of unutilated tissues, even in advanced stages of decomposition and when the intercellular spaces were crowded with the organisms.

Careful tests have been made at various times and with cultures in different media for evidence of diastase (amylase) but none has been found.

This action upon the cell membranes was evidently of an enzymic nature. The chief task we set before ourselves was to isolate this enzyme from the living organism and study its characteristics. Five methods have been tried for accomplishing this separation: heat, filtration, the use of germicides, diffusion, precipitation with alcohol.

The first three of these methods have involved in all cases alike the following procedures: The cultivation of the organism in beef or vegetable broths for several days; treatment of such broth cultures aiming to kill or remove the bacilli without injury to the enzyme; determination by trial transfers of the sterility of the broth so treated; in case sterility was proved, the testing of its cytolytic activity by immersion in it, under sterile conditions, of vegetable tissues. In all cases control trials were made with the uninoculated broths or solu-

tions of the chemicals under trial to preclude the possibility of cytolytic action by these alone.

We will now very briefly summarize the results obtained by each of these five methods, leaving further details for elucidation in our later publication.

Separation of the enzyme from the living bacteria by heating.

Broth cultures of *Bacillus carotovorus* are sterilized by ten minutes exposure to a temperature of 51° C. Previous students¹⁾ of cytolytic enzymes have found them to be destroyed at about 60° C. It seemed probable, therefore, that by heating broth cultures to some intermediate temperature one might kill the bacteria and leave the enzyme active. This was attempted in a series of tube cultures, 10 ccm each, heated by immersion in a water bath. The results were uniform and satisfactory. Cultures so exposed at each 54, 55, 58, 60, 62, 63, 64, 65, 68, 73° were sterilized. Subsequent trial showed that cytolytic activity remained in the broths so heated until 62° was passed when it was lost. There was however some weakening of the cytolytic action by such heating at 54° and much more at 58° . Slight activity persisted in broths heated at 60° , and a trace at 62° . Above this there was none. In brief then there was slight inhibition of activity as a result of such heating for ten minutes at 54° , decided inhibition at 58° , and total inhibition at 63° . A further discussion of temperature relations occurs later in this article.

Separation of the enzyme from the bacteria by filtration.

The Pasteur-Chamberland filter was used and the usual precautions taken to prove the continued sterility of the filtrate. In all our trials cytolytic activity has been found in the sterile filtrate, and the action upon vegetable tissues was indistinguishable in character from that occurring in the presence of the living organism. The rate of this cytolytic action in the filtrate was however decidedly less than that in the original broth. In some cases this loss was estimated at 90 per cent, but in none was it complete.

These results may profitably be compared with those of Potter²⁾, Laurent³⁾ and Van Hall⁴⁾, each of whom found similar bacterial filtrates to retain their cytolytic activity, whereas Spieckermann⁵⁾ found the sterile filtrate from cultures of his kalerot organism to have entirely lost its enzymic activity. If one seeks an explanation of this loss through filtration it may be attributed to either of two things, first, the retention of only that portion of the enzyme which is still closely associated with the organisms, i. e. within the cells or upon their surfaces; or, second, the further removal of enzyme which has

1) Cf. Green, loc. cit. p. 98.

2) Potter, M. C., On a bacterial disease of the turnip. (Proc. Royal Soc. London. Vol. LXVII. 1900. p. 448.)

3) Laurent, E., Recherches expérimentales sur les maladies des plantes. (Ann. Inst. Past. T. XIII. 1899. p. 1.)

4) Van Hall, C. J. J., *Bacillus subtilis* und *B. vulgatus* als Pflanzenparasiten. (Centralbl. f. Bakt. Abt. II. Bd. IX. 1902. p. 642.)

5) Spieckermann, loc. cit.

already passed from the organisms into solution in the broth. The latter theory is favored by Friedenreich's observations which also aid in reconciling the apparent discrepancies between Spieckermann's results and those obtained by ourselves and others. Friedenreich¹⁾ working with cheese extracts found that porcelain filters might retain as high as 90 percent of the protein matter which was in solution and that the amount so retained was much greater with bougies which had been used repeatedly than with new ones. In some of his trials the milk enzyme galactose was entirely removed by filtration.

Separation of the enzyme from the living bacteria by germicides.

In our attempts to accomplish this use has been made of chloroform, phenol, thymol and formalin. Trial additions of various amounts of each of these have been made to broth cultures of the carrot-rot bacillus and the effects noted upon the life of the organism and the activity of the enzyme.

Formalin. Merck's formalin was used in all cases. It has been found that both the bacillus and the enzyme are extremely sensitive to this chemical. Since, however, the bacillus is slightly more so, it is possible, by carefully regulating the amount, to sterilize the broth and leave the enzyme active. The addition of 0,1 percent of formalin to beef broth tube cultures of *B. carotovorus* sterilizes, providing there is thorough agitation. More formalin, viz., 0,2 percent or more, may be necessary to sterilize if the agitation is less thorough. Complete inhibition of the enzymic (cytolytic) activity in such culture broths occurs where 0,6 percent or more of formalin has been added. Amounts as low as 0,3 percent retard the action to a marked degree and retardation was perceptible when even 0,06 percent was used, although this is not enough to sterilize with certainty. Trials where the formalin was added to aqueous solutions of the enzyme-containing alcoholic precipitates (see discussion later) gave results in accord with the above, viz., a slight but appreciable retardation from additions of 0,5 percent and almost complete inhibition where 0,5 percent was used.

In all of the above cases the determinations of enzymic activity were made a day or more after the formalin was added to the broth. After the completion of these trials Spieckermann's²⁾ report came to hand in which he states that 0,2 percent of formalin sterilized cultures of his kale-rot organism and did not inhibit the action of the enzyme, at least for several hours. This statement led us to repeat our observation with the carrot-rot bacillus that we might learn the relative rapidity of the injurious action of formalin upon each the organism and its cytolytic enzyme. The details must await our later publications. The action was as Spieckermann had observed, more rapid upon the bacillus than upon the enzyme. Thus, 0,2 percent of formalin caused appreciable injury to the organism at the end of about three hours, as shown by delay in the growth when transfers were made from such treated broths. The lessening of cytolytic activity in such

1) v. Friedenreich, E., Landw. Jahrb. der Schweiz. Bd. XIII. 1899. p. 169. Ann. Agr. Suisse. T. I. 1900. p. 77.

2) Spieckermann, loc. cit. p. 166—167.

broths became apparent only after a longer delay, viz., nine hours more or less.

These results showed that formalin cannot be employed as an agent for the purposes we wished since if one makes the studies within a few hours after the additions of the formalin sterility of the culture broths is not insured; if, on the other hand, one waits much longer the activity of the enzyme may be impaired.

Bliss and Novy²⁾ have shown that fibrin which has been acted upon for a short time by formalin resists thereafter the action of proteolytic enzymes. Their observations led us to wonder whether any part of the retarding influence of formalin observed in our experiments might be due to similar action of the formalin upon the cell walls of the vegetable tissues rather than upon the enzyme. Trials showed that this is not the case, since vegetable tissues immersed in formalin and later washed in water were decomposed in the presence of the enzyme as readily as were fresh tissues.

Phenol. Numerous trials of this gave satisfactory results. A crystal about one-half the size of a pea added to a 10 ccm broth culture, i. e. making approximately 0.5 percent solution, well agitated, has in all cases secured sterility and caused no appreciable lessening of the activity of the enzyme.

Thymol. This has given variable results, sometimes sterilizing and sometimes not. These irregularities are, we believe, due to the slight solubility and slow diffusibility of thymol in the broth. It was found that powdered thymol floats on the surface of the broth even though well shaken. This has sterilized the upper portions of broth tubes, when added in excess, while living organism persisted indefinitely, at least for many days, in the depths of such tubes, providing they were not repeatedly agitated. Thorough agitation did, however, secure sterilization where 0.2 percent or more of thymol was used. In no case did thymol cause appreciable lessening of the activity of the cytolytic enzyme.

Chloroform. Since this is the agent usually employed for the inhibition of bacterial growths in the study of enzymes, careful trials were made to determine its relation to the activities of both enzyme and bacillus. Here again numerous details must await our later publication. The general results were as follows: The addition to beef broth cultures (the ordinary tube cultures stoppered with cotton) of *Bacillus carotovorus* of as large amounts of chloroform as 50 percent did not sterilize where unaccompanied by thorough agitation, i. e. such cultures may continue to increase in cloudiness and transfers show living organisms for days there-after. In all trials, however, when 10 percent or more of chloroform was added to broth cultures and these were very thoroughly agitated sterility was secured. With less than 10 per cent the results were not constant. In no case has chloroform caused appreciable lessening of the cytolytic action in such broths, nor has it in trials where we have added it to the aqueous solution of the enzyme-containing alcoholic precipitate to be discussed later.

A comparison of the results obtained by other investigators who have tested these chemicals upon similar organisms shows a lack of

1) Bliss, C. L. and Novy, F. G., Action of formaldehyde on enzymes and on certain proteids. (Journ. Exper. Medicine. Vol. IV. 1899. p. 52.)

conformity, especially with chloroform. Smith¹⁾ was the first to emphasize the fact that many bacteria are surprisingly resistant to the germicidal action of chloroform and thymol. Potter²⁾ did not succeed in sterilizing cultures of his *Pseudomonas campestris* by the use of formalin, thymol or chloroform. Spieckermanns³⁾ results with formalin have already been reviewed: he also found chloroform an effective agent for sterilizing cultures of his kale-rot organism and observed no retardation of its cytolytic enzyme thereby. Van Hall⁴⁾, on the contrary, reports that even 0,5 percent of chloroform acting only fifteen minutes destroyed all traces of cytolytic activity in broth cultures of his *Bacillus omnivorus*. These differences are not easily reconcilable. We are inclined to attribute the destruction of the enzyme in Van Hall's exceptional experience to some agency other than the chloroform. In other cases the differences may be explained in part, if not altogether, by differences in manipulation, especially as to amount of agitation. Certainly Smith's conclusions are justified that chloroform cannot be relied upon implicitly as a germicidal agent, as has heretofore been done so frequently in enzyme studies. Rightly used, it has, however, proved the most satisfactory germicidal agent in our studies.

Separation of the enzyme from the bacteria by diffusion.

As already described, the softening of the vegetable tissues occurs several cells in advance of the actual point of invasion of the carrot-rot bacillus. This is also in accord with the observations of several other investigators of soft-rot organisms and indicates that the diffusion of the cytolytic enzyme occurs quite rapidly. Van Hall's⁵⁾ recent experiments with *Bacillus omnivorus* showed this to occur through layers of sterile agar. We made similar trials with cultures of *B. carotovorus* and secured like results.

The method as we developed it consisted in implanting the organism in the middle of a poured plate of stiff (2 %) nutrient-broth agar, the agar layer being about 5 mm thick. On the third day, when the colony was about 1 cm in diameter, this sheet of agar bearing the colony was transferred to the sterile surface of a freshly cut slice of living turnip root, with precautions against contamination. Repeated trials have shown a rapid rotting of the turnip tissues underlying the colony with discoloration, death of the protoplasm and cytolytic action exactly as occurs in the presence of the bacteria. Transfers of bits from this underlying rotten turnip tissue have, however, in all cases proved its sterility. The action must therefore be attributed to the soluble and diffusible products of the bacterial colony on the supernatant agar.

Similar experiments in which the agar layer carrying the bacterial colony was superimposed upon the surface of a sterile layer of gelatine have shown a corresponding liquefaction of the gelatine underlying

1) Smith, Erwin F., Growth of bacteria in the presence of Chloroform and Thymol. (Science, N. S. Vol. XIII. 1901. p. 327.)

2) Potter, M. C., Proc. Roy. Soc. Vol. LXVII. 1900. p. 448.

3) Spieckermann, loc. cit. p. 166.

4) Van Hall, Zeitschr. f. Pflanzenkr. Bd. XIII. 1903. p. 129—144.

5) Van Hall, Centralbl. f. Bakt. Abt. II. Bd. IX. 1902. p. 649.

the colony, which again must be attributed to the diffused products, since the liquified gelatine was sterile.

Separation of the enzyme from the living bacteria by precipitation with alcohol.

In this work the broth culture of the desired age was filtered in one or another way (see details below), then 95 percent alcohol was added, the flocculent precipitate collected, dried, and finally redissolved in water as desired for study. In connection with this work several questions arose as to details of methods, and experimental studies were undertaken to decide these. The following is a summary of the results.

Filtration. The alcohol throws down, along with the enzyme, the various proteid contents of the culture broths and the precipitate also carries down the bodies of the bacteria. It is therefore plainly desirable (previous to precipitation) to remove from the broth by filtration such of this other matter as one can without lessening the enzyme content. Filtration through porcelain was followed by Potter in his work. We tried this also in our earlier experiments, but as already explained we found the enzyme content of the filtrate so much reduced thereby that we discontinued it. Thereafter we were content to filter simply through paper which removes only the coarser deposits from the culture broths. There is no objection to the employment of paper filters in the manipulations with this enzyme, since it is inactive upon cellulose.

The most favorable strength of alcohol. *B. carotovorus* is killed by 25 percent alcohol. Even this strength will throw down a considerable precipitate, and trials were made to determine the optimum per cent for securing the enzyme and also to learn whether a purer enzyme could not be obtained by some method of fractional precipitation or reprecipitation. Without here giving details we will merely state that the precipitate secured in the presence of 80 percent alcohol was found to include practically all obtainable by any higher or lower strength, and also that the enzyme so secured showed a maximum of activity. Nor was the enzyme much purified when the precipitate from 80 percent alcohol was dissolved in water and again precipitated with alcohol.

Method of drying. The method commonly recommended for securing enzymes is to wash the precipitate in absolute alcohol and dry in partial vacuum over sulphuric acid. This process has given excellent results in our trials, but equally good have been secured more expeditiously by drying quickly in a gentle current of warm air, not above 40° C. To secure more rapid drying the precipitate should be broken up and occasionally stirred. Spieckermann¹⁾ washed the precipitate in absolute alcohol followed by ether, presumably to expedite the drying. We tried this method and found our enzyme much weakened. Absolute alcohol followed by chloroform, on the other hand, gave an enzyme of full activity but offered no advantages over drying directly from the 95 percent alcohol.

Strength of solution. The dry precipitate obtained from beef broth cultures with 80 percent alcohol has averaged about 0.35 percent

1) Spieckermann, loc. cit. p. 165.

of the weight of the broth. When this is added to water it swells and is partially dissolved, and the solution so obtained shows cytolytic activity of exactly the same character as does the living broth cultures. Such solutions were used much in our investigations and two important questions presented themselves: first, the relation of the dilution of this aqueous solution to its cytolytic activity; second, the relative enzymic activity of such aqueous solutions of the precipitate in comparison with that of the original broth cultures. Here again the details must await our later report and only the general results now be given. Regarding the first question, it has been found that the cytolytic activity increases with the concentration of the solution. Thus where we compared 1 percent, 5 percent and 10 percent additions of the precipitate to distilled water a like amount of cytolytic action was secured in the following periods of time, respectfully: with the 1 percent solution, in 25 minutes; with the 5 percent in 15 minutes; with the 10 percent in 10 minutes. This shows that the enzymic activities of these solutions stood to each other in the ratio of 6:10:15. In our practice we have used the 5 percent solution almost altogether.

The second question is of especial interest since it involves the query as to whether the enzyme is injured by alcoholic precipitation. As already stated, the cytolytic action of the solution, as shown alike by macroscopic and by microscopic appearance, is of the same character as that occurring in the original broth cultures. One might expect some loss or weakening of the enzyme in the process of precipitation, desiccation and re-solution, even if the enzyme is not altered in its essential composition. As a matter of fact, however, our trials have shown no appreciable loss in this way. The precipitate, whether from beef broth or vegetable juice cultures, when brought into an aqueous solution of the same volume as that of the original culture has shown cytolytic activity equalling that. Spieckermann¹⁾ has reported similar results with his kale-rot organism.

The relation of cultural conditions to enzymic production.

1) The composition of the medium. The vigor of development of the carrot-rot organism varies widely in different media, and it is of much interest to learn whether there are corresponding variations in enzyme production. De Bary²⁾ suggested that the disorganization products of the cell walls of the host plant are probably the chief source of food of the soft-rot fungus *Peziza sclerotiorum* and that these are rendered available by the wall-dissolving enzyme. Ward³⁾ concluded that in the physiologically similar lily *Botrytis* the production of this cytolytic enzyme is a starvation phenomenon. This idea is, moreover, in general accord with the conclusion of Brown and Morris⁴⁾ that diastase secretion in germinating barley is excited by cellular starvation. We were led to make various experiments to learn whether there is any constant relation between nutrition and amount of enzyme produc-

1) Spieckermann, loc. cit. p. 166.

2) De Bary, A., Ueber einige Sklerotien und Sklerotienkrankheiten. (Bot. Zeitung. Bd. XLIV. 1886. p. 378.)

3) Ward, H. M., A lily disease. (Ann. of Bot. Vol. II. 1888. p. 319.)

4) Brown, H. T. and Morris, G. H., The germination of some of the Gramineae. (Journ. Chem. Soc. Trans. Vol. LVII. 1890. p. 498.)

tion with the carrot-rot bacillus, and, especially whether the production of this cytolytic enzyme is a starvation phenomenon. In these experiments determinations have been made of the enzyme product from cultures in media varying widely in nutritive elements and especially in carbohydrate content. These have included Dunham's peptone solution with and without the addition of carbohydrates, nutrient beef broth with and without the addition of carbohydrates, cooked vegetables entire and the same filtered after cooking so as to remove the cell wall substance and other insoluble parts, and finally living vegetable tissues.

Only the general results and conclusions can be given here. Cultures in the fresh turnips and carrots made an exceedingly vigorous development and the expressed juice from these decaying vegetables has shown a higher enzyme content than has any other medium. On the other hand, Dunham's peptone solution which is a starvation medium for this organism has shown the least enzyme development. The presence of cane sugar favored the development of the bacillus and likewise it increased the enzyme product. Thus, beef broth with 2 percent sugar showed a more rapid growth of the organism and also a greater enzyme product than the same without the sugar. The presence or absence of cell-wall substance in the cooked vegetable media made no difference in the enzyme production.

The conclusions reached are, first, that enzyme production is directly proportional to vigor of development of the organism: second, that there is no evidence that enzyme production is a starvation phenomenon, but rather the reverse; third, we find no evidence that the products of enzymic activity are used as food by the organism, although our experiments are insufficient to justify final conclusions in the latter point. These conclusions are in general accord with those of R. E. Smith¹⁾ in the case of *Botrytis cinerea*, since he holds that the fungus can only develop its cytolytic enzyme in the presence of abundant food.

2) Age of culture. The enzyme product has been compared in beef and vegetable broth cultures of various ages from one to seventeen days. It has been found that the amount increases with the age of the culture up to a certain point, then remains about constant. This increase of enzyme content corresponds to the rate of development of the cultures as shown by the degree of cloudiness of the broth, ceasing when it passes its maximum. The conclusion already formulated seems again justified that enzyme production by this organism is not a starvation phenomenon but rather the normal accompaniment of vigorous development under favorable nutritive conditions. Moreover, it is evident that the enzyme is a fairly stable compound which, following its excretion into the broth, tends to accumulate with the age of the culture.

3) Temperature. The optimum temperature for growth of this bacillus is 28–30° C, i. e. broth tubes cloud more rapidly at this temperature than when above or below it. We expected, in the light of the results just described, to find this temperature the optimum for enzyme production also. It has been found, however, that cultures at laboratory temperature, 18–22° C, develop a greater amount of enzyme than do parallel cultures grown at a constant temperature of 30° C.

1) Smith, R. E., The parasitism of *Botrytis cinerea*. (Bot. Gaz. Vol. XXXIII. 1902. p. 421–436.)

Relation of various conditions to the activity of the enzyme.

This has been worked out largely with aqueous solutions of the alcoholic precipitate, rather than with living cultures, because of the greater convenience and closer uniformity of trials with such solutions. The relation of strength of solution to its cytolytic activity has already been discussed. The more important of the other matters worked out are as follows:

1) Effect of long keeping. As already stated, the enzyme seems to be a stable compound which persists unchanged for a long time in the culture broths. It can also be preserved indefinitely as the dry precipitate. Repeated trials with such after one or two months keeping have shown no alteration in their activity, and in one case a careful re-trial of such a precipitate two years old has shown no appreciable loss from such long keeping.

2) Temperature. A series of careful comparisons has shown that the aqueous solution of the precipitated enzyme exercises very little cytolytic action at 2° C; the activity is good at 20°, better at 32°, best at 40—45°. At 48° it is considerably inhibited, and it is wholly checked at 51°. Thus, action was practically twice as rapid at 42° as it was at 22°, and at 32° it was about midway in activity between the higher and the lower degrees. In these temperature relations therefore it is similar to the cytolytic enzyme of germinating barley as recorded by Brown and Morris¹⁾. Holding at 49° for an hour's time was not injurious to the enzyme, i. e. although inhibited while at that temperature, it resumed normal activity thereafter as the temperature was lowered. When, however, the temperature was carried to 51° or above the enzyme was destroyed in such solutions.

In this connection it may be recalled that the trials, discussed earlier in this article, showed that in the original broth cultures the point of thermal destruction is some ten degrees higher. Similar variations in the point of thermal destruction under changed conditions have been recorded for other enzymes²⁾. Thus, invertase withstands a temperature twenty-five degrees higher when in the presence of cane sugar, upon which it acts, than in its absence. Woods³⁾ has shown that the oxydizing enzymes of the maple leaf withstand a higher temperature when in the juices of the plant than in the presence of alcohol. Thinking that the presence of the vegetable tissues upon which it is active might restore to our precipitated enzyme its ability to withstand the higher temperature, comparative trials were made at 51° where pieces of carrot were immersed in the solutions during the heating process. The enzyme was nevertheless destroyed, alike in the presence or the absence of the vegetable tissue.

3) Effects of alkalies and acids. Our earlier studies showed this bacillus to be a facultative parasite upon various vegetables, all of which have an acid cell sap. In the course of its invasion the reaction of the vegetable juice is rendered alkaline. It is of interest, as bearing upon the question of parasitism and resistance thereto, to learn the

1) Brown and Morris, loc. cit. p. 502.

2) Green, loc. cit. p. 448.

3) Woods, A. F., The destruction of chlorophyll by oxydizing enzymes. (Centralbl. f. Bakt. Abt. II. Bd. V. 1899. p. 745—754.)

relation of the reaction of the plant juices or other solvent to the activity of the enzyme. For learning this the alcoholic precipitate, which is neutral, was used. The relative cytolytic activity of this upon carrot tissues was determined when it was dissolved in water along with various acids or alkalies. In all the following discussion the strength of these is given as determined by titration with phenolphthalein against normal solutions.

Alkali. It was found that sodium hydroxide titrating — 2,0 percent inhibited slightly and that this inhibition increased with increase of alkalinity up to — 10,0 percent when it was total.

Acids. A very small addition of hydrochloric acid favored the enzymic activity, about +0,5 percent being the optimum. The difference between this and the neutral solution was, however, but slight. When the reaction reached +2,5 percent inhibition was practically complete.

Various organic acids were tested in like manner, including oxalic, acetic, formic, tartaric, malic and citric. In no case did any of these favor the activity. When the titration strength was below +0,5 percent they were practically without effect. Strengths of +1,0 percent and above distinctly inhibited in all cases, and from +5,0 percent to +10,0 percent caused total inhibition. It should, of course, be borne in mind that this degree of acidity as expressed in titration percentage is in all cases a mild one, amounting to 0,5 percent and less when expressed gravimetrically.

4) Relation of plant juices. The freshly expressed juice of some of the most susceptible vegetables was used as a solvent for the enzymic precipitate and these solutions compared with those in pure water. It was found that in all cases tried these juices, which are, of course, acid, lessened the cytolytic activity as compared with the water. Thus, the juice of carrot, titrating +2,0 percent, and of radish, titrating +0,75 percent, both slightly retarded the enzymic action, and there was still appreciable retardation when these juices were diluted to one-half strength with water. The juice of ripe tomato which is more strongly acid, +5 percent, was more decidedly inhibitory, reducing the enzymic activity to approximately one half of that in the aqueous solution, i. e., there was as complete cytolytic action at the end of fifteen minutes in the aqueous solution as occurred in one-half hour in the solution in tomato juice. This retardation was lessened when the tomato juice was diluted with one-half water.

5) Relation to other bacterial products. As a result of his observation upon the bacterial soft rot of the turnip, Potter¹⁾ suggested that the oxalic acid produced by his organism, *Pseudomonas destructans*, may play some part in the dissolving of the middle lamella. Our results, as given above, show that neither oxalic nor any other of the acids tested so function with the carrot-rot organism. Indeed this produces no oxalic acid. It does, however, produce a small amount of some unidentified organic acid, especially in media rich in sugar. The following experiments were made to determine whether this acid or any other product of the bacterial metabolism favors the cytolytic action. Culture broths of various kinds in which the organism had developed were heated to 80° C, which at the same time sterilized them and destroyed their enzyme content. These were then used as solvents of

1) Potter, Proc. Roy. Soc. Vol. LXVII. 1900. p. 451.

the enzymic alcoholic precipitate and the cytolytic activity of such solutions was compared with solutions in pure water. More or less inhibition was apparent in every case where a culture broth was used as the solvent as is shown in the following tabular summary of results.

Nature of solvent	Its reaction	Effect on enzyme
Carrot broth, 12-day culture	slightly alkaline	slight inhibition
Beef broth, 7-day culture	" "	more inhibition
Dunham solution, 16-day culture	neutral	much inhibition
Dunham solution plus 2 per cent cane sugar, 16-day culture	acid	greatest inhibition

There is no evidence here that the acid or other products of bacterial origin aid in the cytolytic action of the enzyme; indeed, the contrary seems indicated. It is worthy of recall in this connection that the chief cytolytic action occurs in advance of the actual invasion by the organism and where the enzyme has passed by diffusion beyond the zone where there is any such accumulation of bacterial products as were present in the culture broths used above.

The cytolytic action of *Bacillus carotovorus* compared with that of various other organisms.

At the beginning of this article attention was called to our conviction that this carrot-rot bacillus is only one of a large number of closely related organisms capable of functioning similarly as wound parasites. Since our earlier publication we have continued studies upon this class of organisms in cooperation with Messrs. H. A. Harding and F. C. Stewart of the Geneva, New York, experiment station, and W. J. Morse of the Vermont station. The full results of these studies will appear soon as a joint publication from these two stations. Previous to this publication it will not be feasible to discuss the details. We will merely state that we have had under observation some forty strains of organisms isolated in one or the other of these laboratories from the rotting tissues of cabbage, turnip or *Amorphophallus simlense*, and in addition five organisms from other sources, as follows: Harrison's *Bacillus oleraceae*, a cauliflower-rot organism from Ontario; Spieckermann's organism of the soft-rot of kale from Germany; Van Hall's two iris-rot organisms from Holland, *Bacillus omnivorus* and *Pseudomonas iridis*; and an organism sent us from Král's laboratory, Prag, as *Pseudomonas destructans*, Potter's white soft-rot of turnip from England. This latter as we have it proves to be a bacillus, and therefore cannot be Potters' original organism.

Comparative trials have shown that these soft-rot organisms, although from different vegetables and widely separated regions, are remarkably similar in enzymic activity. The only exceptional one is *Pseudomonas iridis*, and this as we have it is non-pathogenic. The others all induce similar soft-rots of various vegetable tissues, and from cultures of each a cytolytic enzyme has been secured indistinguishable in kind from that produced by *B. carotovorus*. The only differences are minor ones in quantity of enzyme production or degree of activity shown. In all alike the action consists in solution of the middle lamella, and stops

short of the complete solution of the cellulose layer of the wall (the invasion of the organisms being strictly intercellular). With all alike there is absence of diastatic (amylolytic) action.

These observations are in general accord with the records regarding these and similar soft-rot organisms heretofore published, with the exception of Potter's. He reports the penetration of the walls by *Pseudomonas destructans* and the development of a diastatic ferment. We would again call attention to the fact that we have not his original organism.

While the production of this middle-lamella dissolving enzyme is thus characteristic of a numerous class of bacterial wound parasites, we should note in contrast that Smith¹⁾ has found complete solution of the cell walls to occur in the black-rot of the turnip caused by *Pseudomonas campestris*. This appears, however, to be a slower action of which the details have not been worked out. That we might compare such complete wall solution with the partial solution shown by *B. carotovorus* we repeated Newcombe's experiments with Taka-diastrase²⁾. Our results were in close agreement with those reported earlier by him, showing a rapid solution of the inner or cellulose lamellae of parenchymatous walls followed ultimately by a slower solvent action upon the middle lamella or pectic layer. This action is, therefore, quite different from that occurring with *B. carotovorus*.

Classification and nomenclature of cytolytic enzymes.

In connection with these studies we have been led to compare the numerous accounts of cytolytic action in literature and to speculate as to the evidences of relationship or difference between the enzymes causing these. Until quite recently it was contended that cellulose fermentation might in some cases be attributed to diastase, i. e. that the distinction between cytolytic and amylolytic fermentation was a doubtful one³⁾. Our results have contributed evidence, if such were needed, to establish the correctness of Newcombe's⁴⁾ conclusion that where these two classes of carbohydrate-fermentation occur together, as in germinating barley and with Taka-diastrase, it is attributable to the occurrence in mixture of two distinct enzymes. Instead of classing the wall-dissolving with the starch-dissolving enzymes the present evidence points rather to the need of more clearly recognizing subdivisions of the "cellulose-enzymes" or "cytases" as they are termed. A fuller understanding of the chemistry of the cell membranes must, of course, precede any such a subdivision that is to be permanently satisfactory. For the present we can, however, recognize the following well-defined groups of elements in the so-called cellulose walls: 1) true celluloses; 2) hemicelluloses; 3) pectic compounds.

Omelianski⁵⁾ has recently shown that even the most resistant of the true celluloses may be dissolved by bacterial action, and he con-

1) Smith, Erwin F., The effect of black rot on turnips. (U. S. Dept. Agric. Bureau plant industry. Bull. 29. 1903.)

2) Newcombe, F. C., Cellulose enzymes. (Ann. of Bot. Bd. XIII. 1899. p. 49-81.)

3) Cf. Grüss, J., Landw. Jahrb. Bd. XXV. 1896. p. 385; Reinitzer, F., Hoppe-Seyler, Zeitschr. f. physiol. Chem. Bd. XXIII. 1897. p. 175.

4) Newcombe, F. C., loc. cit.

5) Omelianski, W., Ueber die Gärung der Cellulose. (Centralbl. f. Bakt. Abt. II. Bd. VIII. 1902. p. 193. etc.)

tends that the term "cellulose fermentation" should be applied only to this. The commoner forms of so-called "cellulose-fermentation" really involve only the solution of the second or third of the above groups. Green¹⁾ stated some three years ago his conclusion from the evidence adduced by Newcombe that the enzymes which act primarily on the middle lamella (pectic compounds) of the wall are of a different class from those acting upon the other portions (hemicelluloses). The logical conclusion to date therefore, is that we must recognize three clearly definable enzymes or enzyme-groups each capable of action primarily upon one of the above-named groups of wall elements. If this is accepted then it will conduce to clearness if we differentiate these by names.

The enzyme of *B. carotovorus* and the related soft-rot bacteria is an example of those acting strongly upon the pectic compounds but not capable of hydrolyzing either the hemicelluloses or the true celluloses. Following the customary terminology, pectase would be the most appropriate name for this, but unfortunately it has long been used to designate Fremy's clotting enzyme. If one were to accept Cross and Bevan's²⁾ term "pecto-cellulose" for these pectic elements of the wall then preference might be given to the name "pecto-cellulase" for the enzyme acting on them. The sufficient objection to this is that the term applies to the hypothetical compound of the non-hydrolyzable cellulose elements with the pectic elements which pass into solution under the influence of this enzyme. The name, if adopted, would suggest an enzyme active upon both of these groups.

The name we would adopt is that suggested by Bourquelot and Herissey³⁾, pectinase. It may be objected that they originally applied this name to the enzyme found in barley malt which so changes pectine that it cannot thereafter be clotted by Fremy's enzyme, pectase.

But Bourquelot⁴⁾ later showed that this same extract hydrolyzes the pectic clot, and inferring that the action is due to the same enzyme he extended the conception of the term. This later application of it to the enzyme capable of hydrolyzing the pectic coagulum, including the pectic elements of the cell wall, appears to us to justify its adoption in this sense. A further argument for this position is that its use in this broader sense is finding acceptance with some recent writers.

As already stated, the hydrolysis of the hemicellulose layers of the

1) Green, loc. cit. p. 105.

2) Cross, C. F. and Bevan, E. J., *Cellulose*. London 1895.

3) Bourquelot, E. et Herissey, H., Sur l'existence dans l'orge germe d'un ferment soluble, agissant sur la pectine. (Compt. rend. T. CXXVII. 1899. p. 191.)

4) Bourquelot, E., Sur la pectase. (Journ. pharm. et chem. T. IX. 1899. p. 56.) After the above was written a publication was received from Beijerinck and van Delden, on the Bacteria which are active in flax-rotting (English reprint of paper read at Meeting of Jan. 20, 1904, of Koninklijke Akademie van Wetenschappen te Amsterdam). In this the authors conclude that the rotting is due to a bacterial enzyme acting on the pectose layers of the cell walls, and which is probably identical with the enzyme we have studied. They propose the new name "pectosinase" for this enzyme on the ground that it is not identical with the "pectinase" of Bourquelot and Herissey (footnote, p. 7). In a personal letter of August 16, 1904, Prof. Beijerinck states that he now thinks this is "possibly only a more concentrated solution of the malt-enzyme called cytase by Brown, Morris and Escombe, and pectinase by Bourquelot and Herissey", although he adds "the difference is obvious and unexplained by physical influences, etc."

wall was more rapid with Taka-diestase than that of the pectic layer. We believe the evidence adduced by Newcombe must lead one to consider this action as due to the occurrence in the Taka-diestase of a smaller amount of the enzyme pectinase in mixture with a larger amount of another enzyme which acts primarily on the hemicellulose elements of the wall. Accepting this conclusion, what name should be applied to the latter?

The one used by Oppenheimer¹⁾, cellulase, is too general, since it implies action on all classes of cellulose. The enzyme under discussion acts only on the hemicelluloses, and therefore hemicellulase is the preferable name for it. This is self-explanatory and leaves the name cellulase to be applied either in a general way to all cellulose enzymes, or, as seems preferable to us, reserves it for application to the enzyme capable of hydrolyzing true cellulose which Omelianski has recently shown to exist.

The tendency is now to use the words pectinase and cellulase in a vague way as synonymous with the term cytase, i. e. as applicable to cytohydrolytic enzymes in general. If they be restricted to the more exact usage defined above it leaves the words cytase and cytolyt as convenient and satisfactory terms for use in the broader sense to include in a general or indefinite way all enzymes capable of hydrolyzing the cell walls.

1) Oppenheimer, C., *Ferments and their action*. (Eng. ed.) 1901. p. 187.

